

## NASA GSRP Final Report for Vanessa Lancaster

**Grant Title:** The Protective Roles of the Antioxidant Enzymes Superoxide Dismutase and Catalase in the Green Photosynthetic Bacterium *Chloroflexus aurantiacus*.

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**Dissertation Title:** The Antioxidant Enzymes of the Thermophilic Photosynthetic Green Bacterium *Chloroflexus aurantiacus*.

**Current Employment:** Currently doing a postdoc at Arizona State University, Bertram Jacobs Lab, School of Life Sciences. Study of poxviral vaccines.

### Study Objectives

The purpose of this study was to examine the biochemical response of the green thermophilic photosynthetic bacterium *Chloroflexus aurantiacus* to oxidative stress. Lab experiments focused primarily on characterizing the antioxidant enzyme superoxide dismutase and the response of this organism to oxidative stress. Experiments in the field at the hot springs in Yellowstone National Park focused on the changes in the level of these enzymes during the day in response to oxidants and to the different types of ultraviolet radiation.

### Background and Significance

Living organisms are constantly bombarded by cell damaging reactive oxygen species (ROS). ROS include superoxide anions, hydroxyl radicals, and hydrogen peroxide. ROS's are produced by ionizing radiation, as by-products of normal electron transport processes, and by metal catalyzed oxidation systems. Modifications of proteins, DNA, and lipids by ROS are known to be involved in many pathologies that can result in cell damage and death (1).

The evolution of efficient defense mechanisms against ROS may have been key to the development of life on Earth. Models of early Earth's atmosphere has suggested that hydrogen peroxide was formed by the photolysis of water vapor and then localized in surface waters such as lakes and basins (2). In surface waters today, the primary source of hydrogen peroxide is the interaction between ultraviolet radiation and dissolved organic carbon (3). Early anaerobic photosynthetic organisms were pressured to evolve defense mechanisms against reactive oxygen species. Aerobic and anaerobic bacteria thought to have existed early in the evolution of life have been shown to contain such defense mechanisms (4).

Organisms have developed mechanisms to combat damage from reactive oxygen. These protective pathways include antioxidant enzymes, vitamins, and damage repair pathways. The roles of the antioxidant enzymes superoxide dismutase and catalase in the

green photosynthetic bacterium *Chloroflexus aurantiacus* are the focus of this study. Superoxide dismutase enzymes (SOD's) catalyze the conversion of superoxide anion radicals to hydrogen peroxide and molecular oxygen, while catalases convert hydrogen peroxide to molecular oxygen and water.

*Chloroflexus aurantiacus* is an anoxygenic green gliding photosynthetic bacterium that is prominent in alkaline hot springs at temperatures between 52 and 60°C. This thermophilic bacterium can often be found growing in mats in the hyperoxic environment beneath cyanobacteria and is thus subject to extreme oxidative stress. This is an evolutionarily important organism since it is in the earliest branch of the eubacteria that are capable of photosynthesis and many of its characteristics can be found in other diverse groups of phototrophic bacteria (5). Phylogenetic studies will be critical to determine how the antioxidant enzymes of this early photosynthetic bacterium relate to those found in more complex organisms.

## **Research Results**

### **I. Characterization of SOD:**

Superoxide dismutase from the thermophilic anoxygenic photosynthetic bacterium *Chloroflexus aurantiacus* has been cloned, purified, and characterized. This protein is in the manganese and iron containing family of superoxide dismutases and exhibits the ability to use both manganese and iron catalytically. This appears to be the only soluble superoxide dismutase contained in *Chloroflexus aurantiacus*. Iron and manganese cofactors were identified using electron paramagnetic resonance spectroscopy and were quantified by atomic absorption spectroscopy. Through the metal enrichment of growth media and metal fidelity studies, the enzyme was found to be most efficient with manganese incorporated, yet retained up to 30% activity with iron. Assimilation of iron or manganese ions into superoxide dismutase was also found to be affected by growth conditions. This enzyme was also found to be remarkably thermostable and was resistant to up to 80mM H<sub>2</sub>O<sub>2</sub>. Reactive oxygen defense mechanisms have not been previously characterized in the organisms of the phylum Chloroflexi.

The superoxide dismutase from *C. aurantiacus* was isolated and partially sequenced chemically (6). The gene coding for SOD was cloned and expressed as a fusion protein with maltose binding protein (MBP) in *E. coli*. The 65kDa fusion protein was purified using an amylose column. Pure recombinant SOD was obtained by isoelectric focusing after the MBP-SOD fusion protein was cleaved using the Factor Xa protease. A western blot using antibodies raised against SOD purified from *C. aurantiacus* was performed, which verified the integrity of the recombinant SOD. The monomeric molecular weight of SOD was found to be 23.15kDa by mass spectrometry. The molecular weight of pure native recombinant superoxide dismutase was determined by gel filtration chromatography to be 49.3kDa, indicating that it is a dimer.

The gene sequence of *C. aurantiacus* SOD is 612 base pairs long and its translated protein sequence is 204 amino acids (GenBank Accession Number AY289213). A neighbor joining tree was constructed with the SOD protein sequence from *C. aurantiacus* using bacterial and archaeal SOD protein sequences that have been experimentally determined to contain iron, manganese, or to be cambialistic (Figure 1). This tree shows *C. aurantiacus* SOD grouping with MnSOD's and cambialistic SOD's, which is consistent with the protein sequence analysis and experimental data.

Atomic absorption analysis of MBP-SOD samples demonstrated that  $1.345 \pm 0.045$  atoms of iron and  $0.014 \pm 0.002$  atoms of manganese were present per monomer of SOD, when expressed in the normal *E. coli* system without metal enrichment. Copper, zinc, and nickel were below detection limits. Buffer extracted from each sample using a centricon concentrator, as well as MBP cleaved from SOD and purified by IEF, did not contain measurable amounts of Mn, Fe, Cu, Zn, or Ni.

Electron Paramagnetic Resonance (EPR) spectroscopy confirmed the presence of coordinated iron and some manganese in SOD, Figure 2A and B. Iron was the primary metal present in this SOD. The EPR-active form of iron (using standard EPR methodology) is Fe (III), and the features near 1570 Gauss (about  $g = 4.3$ ) in Figures 2A and B are characteristic of high-spin ( $S = \frac{1}{2}$  ferric iron in a strongly rhombic environment). Adventitiously bound Fe (III) can produce similar spectra. However, the retention of SOD activity in the presence of diethylenetriaminepentaacetic acid (DTPA-Sigma), an effective chelator of iron, along with this enzyme's ability to replace Fe (III) with Mn (III) in manganese-enriched growth media (*vide infra*), indicates that the  $g = 4.3$  absorption is not due to nonspecifically bound iron. Since Mn (III) is incorporated into the active site, it is EPR silent, and therefore is not found in the spectra of the native samples (Figure 2A and C). The characteristic hydrated Mn (II) spectrum is only found when Mn (III) is released from the protein active site upon acidification of the sample (Figure 2B and D).

To determine if *C. aurantiacus* SOD can coordinate manganese instead of iron, *E. coli* expressing the *C. aurantiacus* SOD gene was grown in media enriched with 1mM  $\text{MnSO}_4$ . SOD was purified as described above and analyzed for metal content. Atomic absorption analysis indicated that this SOD contained  $0.004 \pm 0$  atoms of iron and  $0.199 \pm 0.007$  atoms of manganese per SOD subunit. The EPR spectra indicated that manganese was the sole metal ion in this sample, Figure 2C and D.

The specific activity of various SOD samples was measured using the xanthine-xanthine oxidase based SOD activity assay, as described above, Table I. The iron and manganese containing MBP-SOD purified from the 1mM  $\text{MnSO}_4$  enriched *E. coli* culture was found to have an activity of 683.5 U/mg protein. This is about 3-fold higher than the activity of the primarily iron containing MBP-SOD from the *E. coli* grown in LB broth alone. SOD that was enzymatically separated from MBP and purified by isoelectric focusing had a specific activity of 421 U/mg protein. Since there are more SOD dimers present per mg/protein in this sample than in the MBP-SOD sample, this demonstrated that the presence of MBP does not significantly hinder the activity of SOD.

The effect of metal fidelity on the enzymatic activity of *C. aurantiacus* SOD was investigated by an *in vitro* reconstitution experiment with manganous and ferrous sulfate, Table I. The SOD sample that was reconstituted exclusively in manganese had a 9-fold higher specific activity than the SOD reconstituted in only iron. Some precipitation of protein occurred during the reconstitution of SOD with iron alone and with a 1:10 ratio of manganese to iron. This precipitate was removed by centrifugation and the soluble fraction was assayed for activity. In this case, the specific activity of the MBP-SOD from the *E. coli* culture grown in normal LB broth would be a more accurate representation of the activity of iron substituted SOD from *C. aurantiacus*. The activity of SOD was shown to decrease as manganese was replaced by iron. However, the *in vivo* iron substituted SOD still retained up to 30% of its activity. Apo-SOD retained a minimal

amount of activity, which was probably due to the neutral pH required to make the apo-protein. The samples reconstituted in iron only and manganese only were acidified and found to have no measurable activity. This demonstrated that the free metals in solution are not responsible for the activity.

Native gel activity assays were done to visualize the hydrogen peroxide sensitivity of the purified recombinant SOD, Figure 3. This SOD remained active and was not significantly changed with exposure of up to 80 mM H<sub>2</sub>O<sub>2</sub>, which is typically consistent with MnSOD's. *E. coli* MnSOD and FeSOD were included in the experiment as controls to illustrate the inactivation of iron type SOD's and the natural resistance of manganese type SOD's to peroxide damage (7).

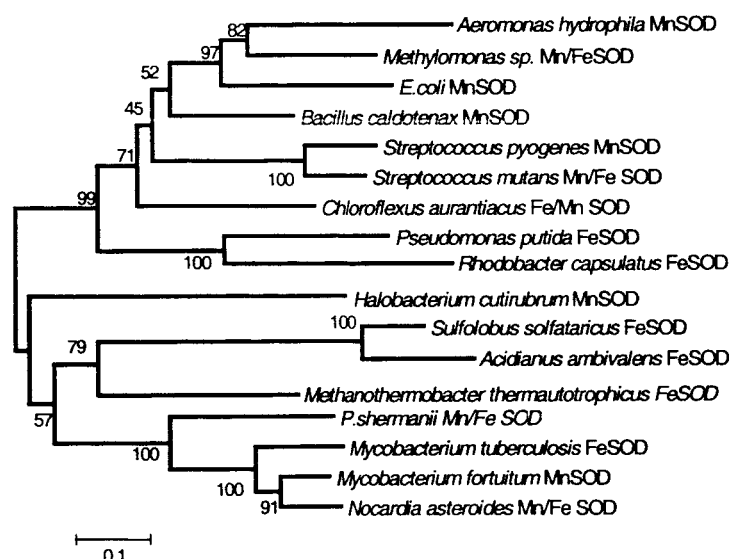


Fig. 1. Neighbor Joining Tree of Experimentally Characterized SOD's from Bacteria and Archaea. The Genbank™ accession numbers for the protein sequences used are: AAL26890, A38461, P00448, CAA44556, CAA11227, P09738, AAC64207, AAA72217, NP\_341862, AAF36989, BAA00489, 1BT8\_B, P17670, CAA50266, AAA91964, NP\_743076.

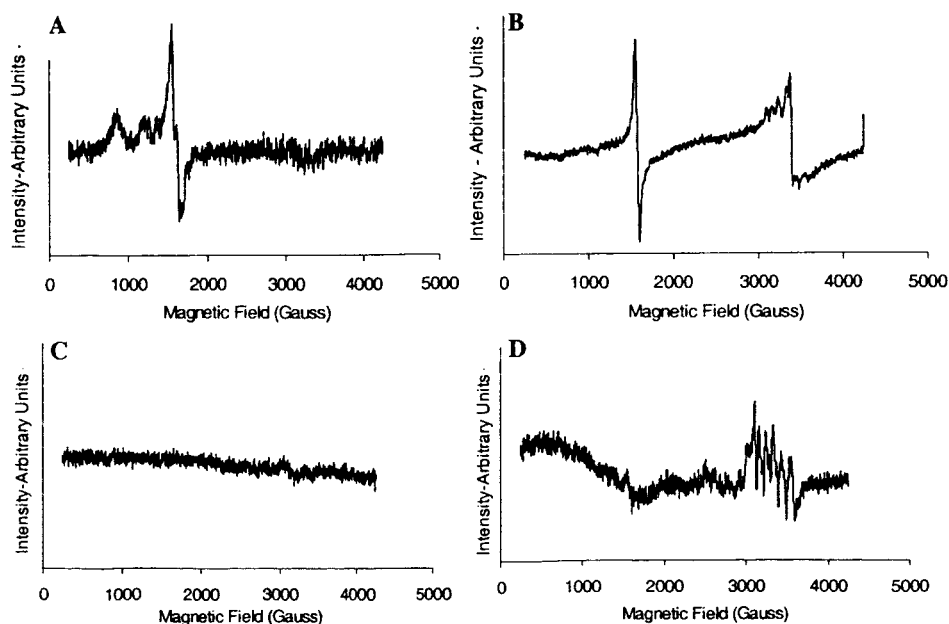


Fig. 2. **EPR Spectra of MBP-SOD.** EPR spectra of MBP-SOD isolated from *E. coli* grown under two conditions. Normal LB growth media: (A) Native MBP-SOD, (B) Acidified MBP-SOD. LB media supplemented with 1mM MnSO<sub>4</sub>: (C) Native MBP-SOD, (D) Acidified MBP-SOD. Iron and, to a lesser extent, manganese are shown in MBP-SOD, and manganese is the only metal detected when *E. coli* is grown in 1mM MnSO<sub>4</sub>.

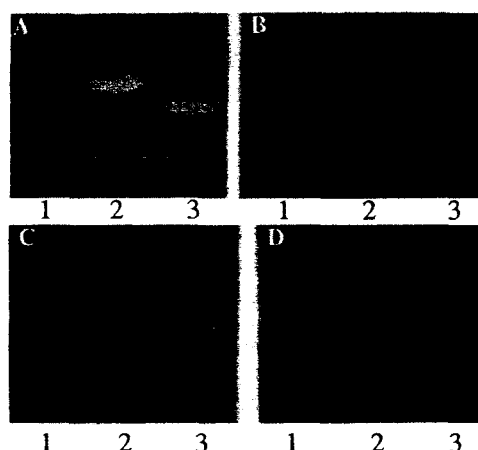


Fig. 3. **Native - PAGE Activity Assays of Pure Recombinant SOD and the Effects of H<sub>2</sub>O<sub>2</sub> Exposure.** SOD samples were run on polyacrylamide gels under native conditions. Each gel is identical except for pre-treatment with H<sub>2</sub>O<sub>2</sub>. Lane 1: 20μg *E. coli* MnSOD. Lane 2: 25μg *E. coli* FeSOD. Lane 3: 5.4μg *C. aurantiacus* SOD. Pre-treatment conditions: (A) Buffer control, (B) 10mM H<sub>2</sub>O<sub>2</sub>, (C) 20mM H<sub>2</sub>O<sub>2</sub>, (D) 80mM H<sub>2</sub>O<sub>2</sub>.

Table I

*Specific Activity of SOD Samples\**

Sample	Specific Activity (units/mg protein)
MBP-SOD	224.0 ± 14.4
Pure recombinant SOD	421.0 ± 28.0
MBP-SOD from 1mM MnSO <sub>4</sub> enriched <i>E. coli</i> media	683.5 ± 68.4
MBP-SOD Apoprotein	11.0 ± 0.01
Mn only reconstitution	572.7 ± 62.9
Mn:Fe 10:1 reconstitution	447.0 ± 44.7
Mn=Fe reconstitution	288.7 ± 18.8
Mn:Fe 1:10 reconstitution	68.0 ± 6.2
Fe only reconstitution	63.0 ± 5.7
Mn only reconstitution, Boiled 5 minutes	511.0 ± 28.4
Controls: Mn and Fe only reconstitution, Acidified	0

\*The specific activity of each sample was determined by the xanthine-xanthine oxidase based SOD assay, as described in "Materials and Methods." One unit is defined as the amount of SOD that reduces the rate of cytochrome *c* reduction by 50%.

## II. Characterization of the Patterns of Superoxide dismutase activity in *C. aurantiacus*

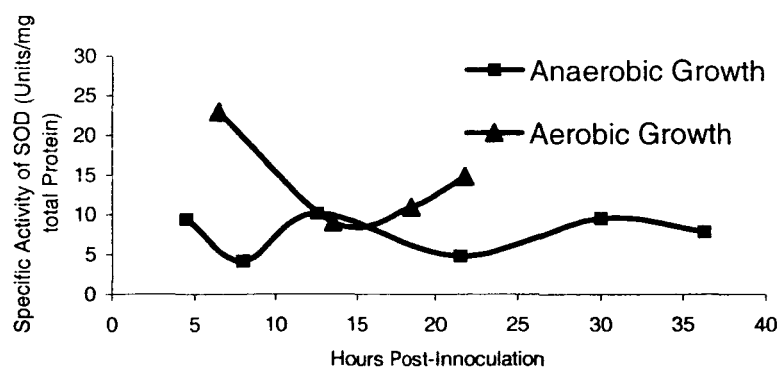
This project focuses on determining whether or not the cambialistic SOD from *Chloroflexus aurantiacus* is inducible in response to oxidative stress. To determine the inducibility of SOD, lab cultures of *C. aurantiacus* were grown anaerobically and aerobically. Aliquots of cells were collected at each stage of each growth curve and analyzed for SOD activity, the amount of SOD present. The amount of RNA transcript produced for SOD is being studied at this time. Comparisons of each of these results will reveal the role that SOD plays in the growth of *C. aurantiacus* and its behavior in an oxygenated environment.

The growth curves for the anaerobically and aerobically grown *Chloroflexus aurantiacus* culture are shown in Figure 4. The density of this culture was monitored by its whole cell absorbance at 650nm. This wavelength was used because it is not in an area of the *C. aurantiacus* whole cell spectrum that is influenced by pigments and is therefore a more true reflection of cell density and not of growth conditions. The anaerobic growth of *Chloroflexus aurantiacus* reaches stationary phase after about 24 hours. This growth curve exhibits two log phases. The first starts at about 8 hours and ends at about 12 hours. A second, less steep log phase occurs from about twelve hours and ends in the stationary phase at about 24 hours. The total log phase lasts about 16 hours. The cause of this two-phase logarithmic growth is unknown, but may be due to *Chloroflexus* switching to an alternative organic substrate (8).

The growth curve of *C. aurantiacus* under oxygenated conditions is slightly shorter than the growth under anaerobic conditions. This culture reaches stationary phase after about 19 hours, and only exhibits a single exponential growth phase. The log phase starts at about 8 hours and ends at about 18, for a total of 10 hours. This log phase is shorter and less steep than that of the anaerobic culture.

The spectrophotometric based activity assays for the anaerobically grown *C. aurantiacus* culture showed approximately the same activity over the course of the whole of the growth curve. Although it appears to oscillate, this data is basically an unchanged baseline of activity. The activity of SOD in the aerated growth curve was drastically different than that of the anaerobic culture. The first time point was taken near the end of the lag phase, when there was enough of a cell density to harvest. The activity here is about 2.5 times higher than the activity of the anaerobic culture. Although active aeration of the media did not yet begin until just after the second time point (14 hours), this culture was still semiaerobic. This 8-liter culture was inoculated with 800mls of an anaerobic culture, and quickly became semiaerobic due to the airspace above the culture. The activity of SOD dropped to the baseline activity level of the anaerobic culture during the exponential phase and then recovered during the aeration of the media.

Anti-SOD western blots for *C. aurantiacus* aerobic and anaerobic cultures were performed. Identical amounts of whole cell extract from each harvested sample were loaded onto two denaturing SDS-PAGE gels. One gel was stained with coomassie blue and the other gel was transferred to a PVDF membrane and developed as a western blot for *C. aurantiacus* SOD. The aerobic culture shows an increasing amount of SOD present as the growth of the culture progresses. The anaerobically grown culture basically shows a steady amount of SOD in each sample.



**Figure 4.** Graphic view of the specific activity of SOD in aerobic and anaerobically grown *Chloroflexus aurantiacus*. Oxygen was bubbled through the aerobic culture directly after the first time point was harvested.

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